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STUDIES ON IMMUNITY TO TOXINS OF *CLOSTRIDIUM BOTULINUM*

IX. IMMUNOLOGIC RESPONSE OF MAN TO PURIFIED PENTAVALENT ABCDE BOTULINUM TOXOID

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Methods for preparation of toxoids from the purified toxins of *Clostridium botulinum* types A, B, C, D, and E, and the immunologic responses of animals to univalent aluminum phosphate-adsorbed toxoids have been described (1–6). With bivalent type AB toxoid, results of studies in animals (6) and trial in man (7) have been presented. The bivalent toxoids were well tolerated and elicited satisfactory responses in man. The percentage of individuals having demonstrable antitoxin titers following the initial series of injections of bivalent toxoid depended upon the immunization schedule. Injections administered at 0, 2 and 10 weeks produced the greatest percentage of responses, and this schedule was selected for routine immunization of man.

Formulation of a pentavalent adsorbed preparation and study of the immunologic responses of rabbits and guinea pigs to the product have been reported by Cardella *et al.* (8). Good responses were obtained to all antigens, but the responses to the pentavalent toxoid appeared to be slightly less than those obtained with univalent control toxoids. The present paper presents an extension of these studies to an investigation of the immunologic response of man to the pentavalent preparation. A preliminary report has been presented (9).

MATERIALS AND METHODS

Four pentavalent toxoids suitable for use in man were prepared by Parke, Davis and Company, Detroit, Michigan, under the direction of Dr. Henry B. Devlin; these products were designated ABCDE-1, ABCDE-6, ABCDE-7 and ABCDE-8. Fluid toxoids of the five types were prepared according to procedures previously described (1–6). Holt's "7/8" aluminum phosphate gel (6) was used as an adjuvant in all preparations. Concentrations of type A, type B, and type D antigens were measured in Lf using univalent antitoxins supplied by the Microbiological Research Establishment, Porton, England. The type E antigen in the first toxoid was expressed in terms of Lf, but difficulty was encountered with the flocculation of the purified type E toxins, and in the last three toxoids the concentrations of type E antigens were expressed in terms of the LD_{50} of the purified toxin before detoxification. The purified type C toxin did not flocculate; therefore the concentrations of all type C antigens were expressed in terms of LD_{50} equivalents also. The five types of concentrated fluid toxoids and saline were added to an aluminum phosphate suspension so that the final product contained 1.7 Lf/ml of type A, 0.54 Lf/ml of type B, 50,000 LD_{50} equivalents of type C, 4 Lf/ml of type D and 0.5 Lf/ml (ABCDE-1) or 100,000 LD_{50} equivalents/ml (ABCDE-6, 7, and 8) of type E. The final aluminum phosphate concentration was 7 mg/ml. A 1% thimerosal solution was added as a preservative prior to the addition of saline, so that the final concentration was 1:10,000. The product was placed on a mechanical shaker at 37°C for 18 to 24 hr and then stored at 4°C. In addition, five univalent control toxoids were produced by an identical procedure simultaneously with pentavalent ABCDE-1 toxoid; these univalent toxoids contained identical antigens at the same concentrations as they were present in the ABCDE-1 toxoid.

Studies of the immunologic response in man to pentavalent toxoids were divided into two parts. The initial experiment was carried out with toxoid ABCDE-1 and its univalent control toxoids and this was followed by an experiment in which the three additional pentavalent toxoids were studied simultaneously. The simultaneous study of toxoids ABCDE-6, ABCDE-7 and ABCDE-8 had two objectives. The first was further evaluation of the response to pentavalent

botulinum toxoids in man; the second, an evaluation of the procedure for production and standardisation of pentavalent toxoids. Each of the three pentavalent toxoids represented a complete repetition of the entire procedure. The growth, purification and detoxification procedures for each product were identical, and the LD_{50} and Lf data collected during these processes indicated that the final purified toxins of the same type were essentially similar with one exception. The type B toxin that, after detoxification, was used in ABCDE-8 contained a similar Lf value but a much lower LD_{50} than the type B toxins used in ABCDE-6 and ABCDE-7.

Individual antigenic responses to each antigen were determined by neutralisation titrations in mice, using the univalent Porton antitoxins as primary reference standards. Purified toxins diluted with two parts glycerine and stored at −20°C were standardised against the reference antitoxins. The units of Porton antitoxin used for the standardisation of the glycerinated toxins were: type A, 0.02; type B, 0.005; type C, 0.02; type D, 0.16; and type E, 0.00125. The neutralising capacities of the units of the five types of antitoxin were different, but at the level of standardisation each antitoxin neutralised approximately 30 LD_{50} of its homologous toxin. These titers will be referred to as the lowest measurable titers. The procedures for standardisation of toxins and determination of serum antitoxin titers were those described previously (6), except that the gelatin-phosphate buffer used as a diluent was adjusted to pH 6.5 for all types.

Porton type A and type B antitoxins were used as primary standards rather than the National Institutes of Health antisera because the former were active in flocculation as well as in neutralisation titrations; the change allowed

TABLE I

Antigenic responses to univalent and pentavalent toxoids

Type	Pentavalent			Univalent		
	12 weeks	52 weeks	8 weeks postbooster	12 weeks	52 weeks	8 weeks postbooster
	Porton units/ml			*Porton units/ml*		
A						
Mean	0.07	<0.02	1.3	0.2	0.02	7.8
Median	0.05	<0.02	0.6	0.1	<0.02	3.0
% Measurable	65	0	100	83	17	100
B						
Mean	0.03	<0.005	0.2	0.1	0.005	0.6
Median	0.03	<0.005	0.1	0.02	<0.005	0.5
% Measurable	82	6	94	83	33	83
C						
Mean	0.3	<0.02	2.9	—*	<0.02	7.2
Median	0.2	<0.02	1.2	0.2	<0.02	6.0
% Measurable	88	6	100	100	25	100
D						
Mean	0.5	<0.2	8.4	3.5	0.3	25.0
Median	<0.2	<0.2	4.8	1.9	0.2	10.6
% Measurable	47	12	100	80	60	100
E						
Mean	0.05	0.007	2.1	—*	0.02	2.3
Median	0.03	0.008	0.8	>0.06	0.02	1.3
% Measurable	94	94	100	100	100	100

* Means could not be calculated because end points were not reached in all titrations.

use of the same antitoxin as a reference standard in both flocculation and neutralization tests. Comparative neutralization titrations have shown that the type A and type B Porton units have approximately ½ the neutralising capacity of the respective N.I.H. units at the 0.02 unit level for type A and the 0.005 unit level for type B.

Statistical analysis of the data was carried out by the rank sum method (10), since titer distribution curves showed a marked asymmetry.

EXPERIMENTAL RESULTS

Toxoids were administered in 0.5-ml amounts given by deep subcutaneous injections in the deltoid region. Approximately 400 individuals were immunized with pentavalent toxoids and no marked local or marked systemic reactions were noted; three individuals showed either a moderate local or moderate systemic reaction. There were indications that the incidence of mild local reactions was somewhat greater with the pentavalent toxoids than with the bivalent products studied previously (7).

Seventeen persons were immunized with pentavalent ABCDE-1 toxoid, and five or six persons per type were immunized with the univalent control toxoids, on a 0-2-10 week schedule. Individual titers were determined 12 and 52 weeks after the initial injection; boosters were given at 52 weeks, and titers were determined 8 weeks after the booster injection.

The data obtained 12 and 52 weeks after the initial injection and 8 weeks after the booster dose are presented in Table I. Antigenic responses to all antigens were found 12 weeks after the initial injection in at least 47% of the individuals. A considerable drop in titer occurred between 12 and 52 weeks, but with the administration of a booster all titers rose above the levels that had been obtained at 12 weeks. Eight weeks after the booster one individual immunized with the pentavalent toxoid did not have a measurable type B titer; all other individuals had titers to all antigens. The median titers for each type ranged from 20 to 640 times the lowest measurable level. Statistical analysis by the rank sum method showed that the individual responses to the pentavalent toxoid were generally not significantly different from the corresponding univalent responses at either the 12-week or the 8-week postbooster bleeding.

Four months after the initial injections of the first group a second group of 15 individuals was immunized with the pentavalent ABCDE-1 toxoid on a 0-2-10 week schedule; a 0.5-ml booster was given 52 weeks after the initial injection. The individuals were bled 2 weeks after the initial series and 8 weeks after the booster. Table II lists the median titers and the percentage of measurable titers obtained; the results obtained with the first group are included for comparison.

TABLE II

Stability of the pentavalent toxoid

Bleeding	Type	Median Titer		% Measurable	
		Study 1	Study 2	Study 1	Study 2
		units/ml			
12 weeks	A	0.05	0.03	65	53
	B	0.03	0.01	82	73
	C	0.2	0.2	88	79
	D	<0.2	<0.2	47	23
	E	0.03	0.03	94	100
8 weeks postbooster	A	0.6	1.3	100	100
	B	0.1	0.3	94	100
	C	1.2	3.0	100	100
	D	4.8	8.5	100	100
	E	0.8	1.7	100	100

TABLE III

The percentage of individuals exhibiting measurable titers following immunization with pentavalent toxoids

Toxoid No.	Bleeding	Per Cent of Persons with Measurable Titers				
		Type A	Type B	Type C	Type D	Type E
	weeks					
6	14	90	93	100	80	100
	52	43	4	25	7	50
	8 Pb[a]	100	100	100	100	100
7	14	97	80	87	60	90
	52	36	4	16	0	20
	8 PB	100	86	100	89	100
8	14	81	56	89	52	67
	52	29	4	13	4	25
	8 PB	100	94	100	100	100

[a] PB, postbooster.

TABLE IV

The mean and median titers of individuals following immunization with pentavalent toxoids

	Toxoid No.	Bleeding	Type A	Type B	Type C	Type D	Type E
		weeks					
Mean	6	14	0.4	0.04	0.5	1.3	0.03
		52	0.02	<0.005	<0.02	<0.2	0.002
		8 PB*	4.1	0.6	2.2	6.5	0.4
	7	14	0.4	0.03	0.3	0.8	0.02
		52	<0.02	<0.005	<0.02	<0.2	<0.001
		8 PB	5.2	0.4	2.7	2.3	0.1
	8	14	0.1	0.01	0.2	0.3	0.01
		52	<0.02	<0.005	<0.02	<0.2	<0.001
		8 PB	1.5	0.06	3.3	3.9	0.1
Median	6	14	0.2	0.03	0.3	0.5	0.02
		52	<0.02	<0.005	<0.02	<0.2	<0.001
		8 PB	2.1	0.3	1.2	2.4	0.1
	7	14	0.2	0.02	0.1	0.5	0.008
		52	<0.02	<0.005	<0.02	<0.2	<0.001
		8 PB	2.1	0.2	0.8	1.2	0.02
	8	14	0.06	0.006	0.1	0.2	0.002
		52	<0.02	<0.005	<0.02	<0.2	<0.001
		8 PB	0.8	0.03	0.6	2.0	0.02

* PB, postbooster.

Median titers for the two groups were approximately the same after the initial series, although the percentage of measurable titers was somewhat lower with the second group. Following the booster, median titers were essentially the same, and all but one individual had measurable levels of antitoxin for all types. Statistical analysis by type showed no significant differences at the 5% level between the responses of the two groups.

Groups of 30 persons were immunized with pentavalent toxoids 6, 7 and 8 on a 0-2-12 week schedule; a 0.5-ml booster was given 52 weeks after the initial injection. All individuals were bled 14 and 52 weeks after the initial injection and 8 weeks after the booster. Antitoxin titers were determined for each type on each serum. The percentages of individuals exhibiting measurable titers following immunization with each of the three pentavalent toxoids are listed in Table III. Two weeks after completion of the initial series a large proportion of the individuals had titers to all types regardless of the toxoid used. All titers declined between 14 and 52 weeks, and only a small portion of the individuals had measurable titers at 52 weeks. A booster given at 52 weeks was very effective, and all except three of the titers were measurable 8 weeks after the booster.

The arithmetic mean and median titers for all types and for each preparation are shown in

TABLE V

Analysis of the significance of difference in antigenicity between ABCDE-6, ABCDE-7 and ABCDE-8

Bleeding	Type	Level of Significance
14 weeks	A	1%
	B	1%
	C	1%
	D	5%
	E	1%
8 weeks postbooster	A	Not significant, 5%
	B	1%
	C	Not significant, 5%
	D	Not significant, 5%
	E	1%

Table IV. In all cases the median titers were less than the mean titers; the means ranged from 1.3- to 5.5-fold the median titers. After the initial series, median titers were from 2 to 16 times the lowest measurable level; after the booster, they ranged from 10- to 100-fold the measurable level.

The results of a rank sum statistical analysis of the response for each type in the three pentavalent toxoids are presented in Table V. Significant differences between the products were observed, and the differences between products were more evident after the initial series than after the booster.

Preliminary consideration was given to the relationship between the antitoxin titer and protection against botulinum intoxication. Since no such information was available for man, experiments were carried out in guinea pigs. The animals were immunized subcutaneously with aluminum phosphate-adsorbed univalent toxoids and challenged intraperitoneally with homologous toxin. Immune groups of each type with mean antitoxin levels of approximately two times the lowest measurable level withstood 10^5 to 10^6 mouse intraperitoneal LD_{50} of homologous toxin.

DISCUSSION

Procedures for the production of a pentavalent type A, B, C, D and E botulinum toxoid suitable for use in man have been developed, and four products have been tested in man. The toxoids were well tolerated, producing only occasional moderate reactions and no severe reactions in man. Each product produced measurable antitoxin titers to each of the five antigens following a three-injection initial series; the percentages of measurable titers ranged from 23 to 100%. A 0.5-ml booster given 52 weeks after the initial injection was highly effective and the percentages of measurable titers following the booster ranged from 86 to 100%.

Comparison of the responses to the five antigens individually with those obtained after administration of the pentavalent combination appeared to indicate that the antigenicity of the toxoid was decreased by combination. Statistical analysis, however, revealed that these apparent differences were not significant in most cases. The difficulty in revealing such differences as may exist is a result of the large range of titers recorded within groups that had received the same antigen; this range was frequently greater than 1000-fold. Because of this large variation, tests with larger groups would be required to detect even rather substantial differences in antigenicity resulting from combination of the antigens. In any event it is evident that any decrease in antigenicity resulting from pentavalent combination is not of a magnitude to prevent practical use of this combination of antigens for immunization of man.

The first human study with pentavalent toxoid ABCDE-1 was initiated after the product had been held at 4°C for 1 year; satisfactory immunization of man was obtained at this time. A second immunization study 4 months later also yielded satisfactory antitoxin titers in man. These experiments indicated that the pentavalent toxoid produced a good response in man after it had been held at 4°C for at least 16 months.

The simultaneous evaluation of toxoids ABCDE-6, ABCDE-7 and ABCDE-8 demonstrated that the laboratory procedures for growth, purification and detoxification of univalent botulinum toxins and their incorporation into pentavalent toxoids were adaptable to large-scale production procedures. All three pentavalent preparations elicited satisfactory responses in man and were considered acceptable products. However, statistical analysis of the data showed that these products produced significantly different antitoxin responses in man to all types after the initial series. The antigens in each product were standardized on the basis of either Lf or LD_{50} equivalents; the significant difference in antigenicity between toxoids demonstrated as anticipated, that neither Lf nor LD_{50} equivalents is a precise measure of the antigenicity of the botulinum toxoids.

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SUMMARY

Purified toxins of *Clostridium botulinum* types A, B, C, D and E were converted to toxoids and adsorbed on aluminum phosphate; four prepara

tions of pentavalent toxoid were investigated in groups of 15 to 30 volunteers. Each pentavalent product contained 1.7 Lf of type A, 0.54 Lf of type B, 50,000 LD_{50} equivalents of type C, 4 Lf of type D, and 0.5 Lf or 100,000 LD_{50} equivalents of type E toxoid/ml. All flocculation potencies are referred to standard antitoxins prepared by the Microbiological Research Establishment, Porton, England. Doses of the first preparation of pentavalent toxoid (0.5 ml) were administered subcutaneously at 0, 2 and 10 weeks; the other preparations were given in the same way except that a 0-2-12 week schedule was used. Booster doses of 0.5 ml were given 1 year after the initial injection. Serum antitoxin titers of individuals were determined 12 to 14 and 52 weeks after the initial injection, and again 8 weeks after the booster, by neutralization titrations in mice. After the initial series of injections a considerable proportion of the individuals developed antitoxin titers for all or most of the five toxins; the proportion of satisfactory titers varied with the type and also with the preparation. Good booster responses were obtained with all preparations, and antitoxin titers to each of the five types were found in 86 to 100% of the individuals immunized. Stability studies with one preparation showed that the toxoids retained considerable antigenicity for at least 16 months at 4°C.

ADDENDUM

The Porton botulinum antitoxins used as primary standards in this paper were established as International Standards at the 35th Session of the Expert Committee on Biological Standardization, World Health Organization. The size of the type E antitoxic unit was decreased by a factor of 10; multiplication of the type E titers expressed in this paper by 10 will convert the titers to the International unit. Titers of the other four types are already expressed in terms of their respective International units.

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